

Short communication

A ronin-like virus associated with mortalities of the freshwater crab, *Eriocheir sinensis* Milne Edwards, cultured in China, exhibiting 'sighs disease' and black gill syndrome

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The freshwater Chinese mitten crab, *Eriocheir sinensis* Milne Edwards, is an economically important species cultured in China during the last 10 years. Culture areas have increased rapidly in inland provinces of China. However, with the development of intensive culture, various diseases have appeared and severely affected crab production. Among these, 'black gill syndrome' (BGS) has caused important economic losses since 1996, particularly in the main culture provinces in China such as Jiangsu, Zhejiang, Anhui and Shanghai. Another major sign of the disease resembles a respiratory problem. Thus, the disease is often called 'sighs disease' (SD) by farmers, because sounds reminiscent of sighs can be clearly heard from affected crabs, caused by slow extrusion of bubbles at night. Sluggishness and anorexia are other non-specific signs. During investigations on this disease we found a virus developing in connective tissue cells showing the general properties of the Roniviridae and designated it EsRNV for '*E. sinensis* ronivirus'. We report here histological investigations, viral ultrastructure and some genomic features of this new crab virus.

Numerous viral diseases have been reported in crustaceans, since the first was noted by Vago (1966) in crabs. There were several reports of crab viral diseases during the 1970s and early 1980s, which included Reovirus (Bonami 1973, 1980; Johnson & Bodammer 1975; Johnson 1984; Mari & Bonami 1988a), Iridovirus (Montanié, Comps & Bonami 1993), Baculovirus (Pappalardo, Mari & Bonami 1986), Picornavirus (Johnson 1978), Herpesvirus (Johnson 1976; Payen & Bonami 1979; Sparks & Morado 1986), Parvoviridae (Mari & Bonami 1988b), Bunyaviridae (Bang 1971; Bonami, Veyrunes, Cousserans & Vago 1975; Hoover & Bang 1978; Corbel, Coste & Bonami 2003) and Rhabdoviridae (Chassard-Bouchaud, Hubert & Bonami 1976; Jahromi 1977; Yudin & Clark 1978, 1979; Johnson & Farley 1980). However, the rhabdo-like virus A (RhVA or EGV-2) of *Callinectes sapidus* (Yudin & Clark 1978, 1979) resembles a ronivirus rather than a rhabovirus on the basis of its size, shape and general structure.

Diseased *E. sinensis* exhibiting typical BGS were collected from a farm in Hubei Province, China. Healthy animals with an average weight of 100 g were purchased from a local farm without a history of disease and maintained in the laboratory for 1 week before experimental infections. The water temperature was maintained at 24–30 °C. Each crab was inoculated at the basal joint of the fifth pereopod with 0.2 mL of haemolymph from a diseased animal, diluted 1/4 in TN buffer (0.04 M Tris, 0.4 M NaCl, pH7.4) and ultra-filtered

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(0.22 μm). Tissues of experimentally infected crabs were collected from days 7–20 post-injection (p.i.).

In naturally infected crabs, most of the gills were dark grey or partly black. Sometimes the whole gill turned dark black when heavily affected. The affected area was most often located at the end of the gill leaflets, but could also be observed at the axis of the gill. Abundant debris was often deposited between the gill leaflets. In all crabs examined, no obvious lack of haemocyte aggregation was observed.

By light microscopy, signs of infection were noted in the lymphoid organs, connective tissues of gills, hepatopancreas (HP), heart and gut. Haematoxylin and eosin (H&E) staining (Bell & Lightner 1988) of infected cells revealed pale to deep basophilic affected areas showing tissue degeneration, abnormal nuclei and dense bodies that could be karyorrhexic and pyknotic nuclei, or inclusion

bodies (Fig. 1a & d). The basophilic areas were approximately 1–5 μm in diameter (Fig. 1a & b). Darkly eosinophilic foci of necrotic cells were located in connective tissue among the normal HP tubules (Fig. 1c), and also in the heart and testis (Fig. 1d). There was a generalized multifocal to diffuse severe necrosis, with prominent nuclear pyknosis and karyorrhexis, in connective cells (Fig. 1d). This apoptosis-like phenomenon is very common during the course of nidovirus infection (Khanobdee, Soowannayan, Flegel, Ubol & Withachumnarnkul 2002). No typical basophilic inclusion bodies were observed in gills. In contrast, eosinophilic affected areas in connective tissue and eosinophilic foci of necrotic cells were observed in the gills (data not shown).

Acridine orange staining (5 mg 100 mL⁻¹ of AO for 20 min followed by phosphate buffer 0.1 M

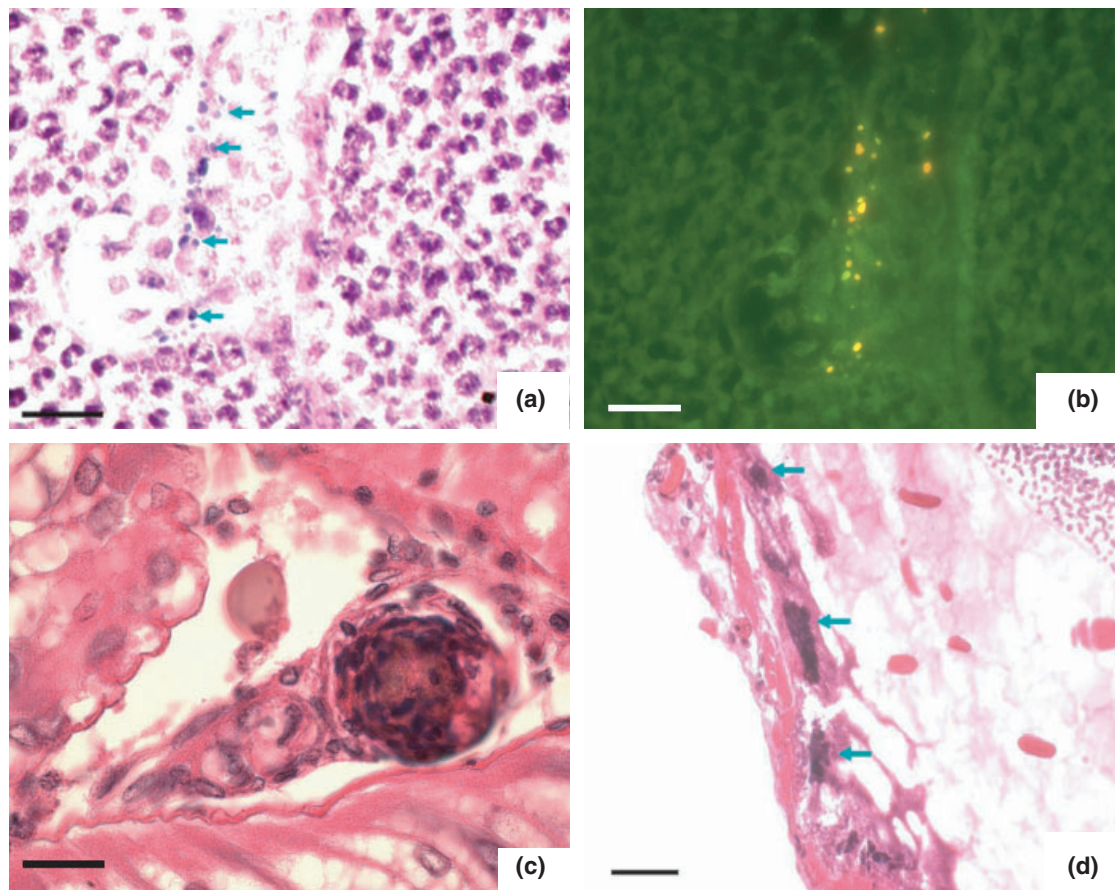


Figure 1 Histological lesions in *Eriocheir sinensis* infected with *EsRNv*. (a) Infected lymphoid organ. Cytoplasm contains basophilic inclusion bodies (arrows) (H&E, bar = 55 μm). (b) Same section as in panel a, but observed under UV. Viral inclusions fluoresce orange-yellow (acridine orange, bar = 55 μm). (c) Eosinophilic area in connective tissue between hepatopancreatic tubules and containing necrotic cells (H&E, bar = 55 μm). (d) Connective tissue of testis with a generalized multifocal to diffuse necrosis, and with prominent nuclear pyknosis and karyorrhexis. Arrows indicate viral inclusion bodies (H&E, bar = 60 μm).

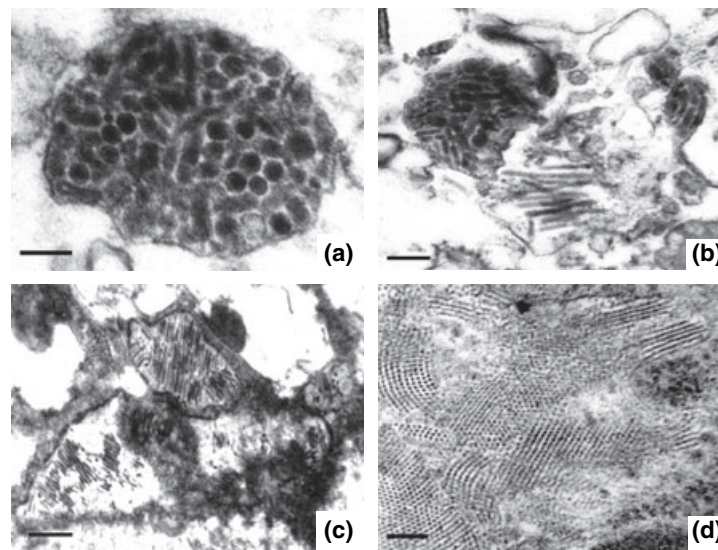


Figure 2 Transmission electron microscopy of connective tissue in *Eriocheir sinensis* infected with *E3RNV*. (a) Large inclusion body in cytoplasm. Mature enveloped virions (seen in transverse and longitudinal sections) are $60\text{--}110 \times 24\text{--}42$ nm (bar = 70 nm). (b) Both rod shaped enveloped virus particles in vesicles and free viral nucleocapsids form viral cytoplasmic inclusions in connective cells of the hepatopancreas. Non-enveloped, filamentous forms measuring up to 400 nm may also be seen in the cytoplasm (bar = 120 nm). (c) Arrays of *E3RNV* nucleocapsids ($150\text{--}250 \times 16\text{--}18$ nm) enclosed within a cytoplasmic vesicle in connective tissue cell (bar = 250 nm). (d) Small particles, 8–10 nm in diameter, in peri-nuclear area in gill connective tissue cells (bar = 250 nm).

sodium phosphate, pH 7.2) revealed green fluorescence in nuclei and bright orange inclusion bodies in the cytoplasm (Fig. 1a & b), suggesting the presence of ssRNA in the inclusions.

Tissue samples were fixed in 4% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide and embedded in Spurr's resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi 7000-FA transmission electron microscope (TEM) (Hitachi, Beijing, China) operating at 80 kV. Enveloped and non-enveloped cytoplasmic rod-shaped particles and filamentous forms were observed in connective tissue cells. The enveloped virions (Fig. 2a & b) were $60\text{--}110 \times 24\text{--}42$ nm, with a 8-nm thick external membrane layer (envelope) containing electron-dense nucleocapsids, 15 nm in diameter. These structures represent mature virions which were randomly and loosely arranged in cytoplasm or packed in inclusion bodies.

The nucleocapsids were usually $150\text{--}250 \times 16\text{--}18$ nm. Some measured up to 400 nm in length (Fig. 2b & c) or were enclosed within membrane bound vesicles forming parallel arrays (Fig. 2c).

Inclusion bodies observed by light microscopy in the cytoplasm were from 200 to 800 nm in size, filled with enveloped virions and were also limited by an 8-nm thick bi-layered membrane (Fig. 2a & b).

Non-mature particles were occasionally found associated with the endoplasmic reticulum. Similar observations were reported in gill-associated virus (GAV) (Spann, Cowley, Walker & Lester 1997). Numerous mitochondria around the virions had lost their internal organization.

In the gills, multiple small particles, 8–10 nm in diameter, were found accumulated to form paracrystalline arrays in the peri-nuclear area. These small particles were usually found in 40–70% of cells, whereas enveloped virions were present in < 40% of cells (Fig. 2d).

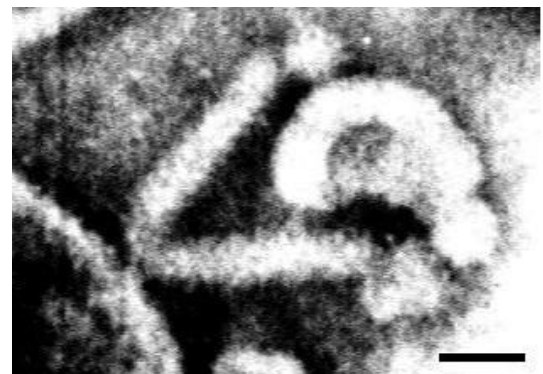


Figure 3 Higher magnification of enveloped *E3RNV* virions partially purified and negatively stained with PTA (bar = 40 nm).

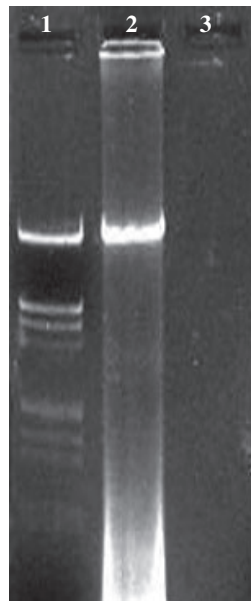


Figure 4 One per cent agarose gel electrophoresis of extracted *EsRVN*-RNA. Lane 1, HindIII-EcoRI λ -DNA digest; lane 2, *EsRVN*-RNA; lane 3, viral RNA digested with RNase.

In supernatant of homogenized infected tissues, particles were observed by TEM after negative staining (phosphotungstic acid [PTA] 2%, pH 7.0). The shape of virions was close to an ellipse of variable length (60–170 nm) and 25–45 nm in diameter (Fig. 3). It was difficult to detect nucleocapsid inside the envelope by this method.

Agarose gel electrophoresis (1% agarose in TAE buffer) of viral nucleic acid, extracted using TRIZOL reagent (Invitrogen) and precipitated with isopropanol, revealed a band of an estimated molecular weight up to 20 kb (Fig. 4). RNase digestion (final concentration $50 \mu\text{g mL}^{-1}$ at 37°C for 1 h) suggested the genome was composed of RNA.

Eriocheir sinensis ronivirus was transmitted in crabs by inoculation of infected tissues into healthy animals. Morphologically identical particles were observed by TEM in experimentally infected animals, confirming *EsRVN* is infectious for *E. sinensis*. Mortalities reached 100% (28 crabs in two tanks) after 17 days p.i., with crabs beginning to die 13 days p.i. No animals died in the control group (seven crabs in one tank). The signs of SD were noted in about 30% of infected animals, however, under laboratory conditions, experiments failed to reproduce BGS. We consider the black gill may be a secondary sign caused by environmental factors after the crabs have been stressed by the viral

infection. Naturally infected animals do not all exhibit BGS, and *EsRVN* was not the only pathogen found in diseased animals. In another farm, a DNA virus in the R-cells of the HP and a proteo-like bacterium were isolated in crabs exhibiting BGS, but were present in only 10% of sampled animals (data not shown). It is not certain that *EsRVN* is the causal agent of BGS, but the virus is undoubtedly very common in cultured crabs in inland China. *EsRVN* does appear to be the aetiological agent of SD as the virus was transmitted by inoculation and the clinical signs were reproduced.

Similar viruses have been found in shrimp, including yellow head virus (YHV) (Boonyaratpalin, Supamattaya & Kasornchandra 1993; Chantachookin, Boonyaratpalin, Kasornchandra, Direkbusarakom, Ekpanithanpong, Supamataya, Sriurairatana & Flegel 1993; Flegel, Sriurairatana, Wongteerasupaya, Boonsaeng, Panyim & Withachumnarnkul 1997; Wongteerasupaya, Sriurairatana, Vickers, Anutara, Boonsaeng, Panyim, Tassanakajon, Withachum-Narnkul & Flegel 1995) from Thailand, GAV (Spann *et al.* 1997) and lymphoid organ virus (LOV) (Spann, Vickers & Lester 1995) from Australia. A yellow head-related virus has also been identified in Taiwan, and outbreaks of yellow head disease have been reported from several other countries in the Asian region. Susceptible host species for yellow head disease include black tiger shrimp, *Penaeus monodon*, Pacific white shrimp, *P. vannamei*, Pacific blue shrimp, *P. stylirostris*, Gulf white shrimp, *P. setiferus*, Gulf brown shrimp, *P. aztecus*, Gulf pink shrimp, *P. duorarum* and Kuruma prawn, *P. japonicus*. The Oka organ (lymphoid organ), gills, heart and subcuticular tissues, including stomach epithelium, contain the highest levels of YHV. Infected cells show nuclear pyknosis and karyorrhexis, which are apparently signs of virus triggered apoptosis (Khanobdee *et al.* 2002).

Yellow head virus and GAV are closely related, but distinct pathogens; LOV is a variant of GAV, which occurs in healthy shrimp (Cowley, Dimmock, Wongteerasupaya, Boonsaeng, Panyin & Walker 1999). YHV and GAV are related to coronavirus and were classified recently in a new taxum (family *Roniviridae*, genus *Okavirus*) within the order *Nidovirales* (Cowley, Dimmock, Spann & Walker 2000; Cowley & Walker 2002; Sitidilokratna, Hodgson, Cowley, Jitrapakdee, Boonsaeng, Panyim & Walker 2002).

Although the morphology of *Es*RVN is like that of roniviruses, it is smaller than YHV and GAV. Enveloped virions are $60\text{--}170 \times 24\text{--}42$ nm, compared with GAV ($183\text{--}200 \times 34\text{--}42$ nm) and YHV ($150\text{--}200 \times 40\text{--}50$ nm). Nucleocapsids are normally $150\text{--}250 \times 16\text{--}18$ nm, compared with GAV nucleocapsids (Spann *et al.* 1997) which are $166\text{--}435$ nm long and $16\text{--}18$ nm wide. Thus, the length of *Es*RVN is much smaller than YHV and GAV, while its diameter is similar.

Although the rhabdo-like virus A (RhVA or EGV-2) of *C. sapidus*, based on its size, shape and general structure, was thought to resemble a ronivirus (Spann *et al.* 1997), it presents some differences from *Es*RVN. In particular EGV-2 particles are longer ($100\text{--}150 \times 25\text{--}30$ nm) and bacilliform in shape.

The infectious character of *Es*RVN and its pathogenesis resembles that of YHV and GAV, with basophilic inclusion bodies in the lymphoid organ and gut and apoptosis in these organs. Eosinophilic foci of necrotic connective cells are also present. These characteristics can be regarded as common to members of the Roniviridae.

The *Es*RVN genome is clearly RNA as it is: (i) extracted with TRIZOL reagent and (ii) is sensitive to RNase. As the virus was located in the cytoplasm and fluoresced orange after acridine orange staining, it may be interpreted as a single stranded RNA virus. S1 nuclease was also tested on the viral genome without a complete digestion of RNA (data not shown), indicating the possibility of at least a partial double stranded structure.

The molecular weight of *Es*RVN was estimated to be larger than 22 kb, which is similar to that of YHV and GAV. Among RNA viruses, the genomic size of Rhabdoviridae and Paramyxoviridae are most similar to *Es*RVN, but their morphology (spherical) is quite different.

Based on the morphological features of viral particles, its infectious character and the nature of the genome, *Es*RVN closely resembles YHV and GAV, but with some obvious differences, and it may be a new member of the Roniviridae.

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